A Mode of Action for Butylated Hydroxytoluene-Mediated Photoprotection

Mark D. Koone, B.Sc. and Homer S. Black, Ph.D.

Photobiology Laboratory, Veterans Administration Medical Center and Department of Dermatology, Baylor College of Medicine, Houston, Texas, U.S.A.

Dietarily administered butylated hydroxytoluene (BHT) has previously been shown to inhibit UV radiation induction of carcinogenesis, erythema, and ornithine decarboxylase (ODC) activity. Butylated hydroxytoluene feeding also resulted in significant increases in epidermal absorption and it was suggested that BHT's photoprotective properties might be attributable to a diminution of UV radiation dose reaching respective target sites. To explore this possibility, the contribution of stratum corneum to BHT's photoprotective action was examined. SKH-Hr-1 hairless mice were fed diets containing 0.5% (w/w) BHT for 2 weeks prior to experimentation. Control animals received the unsupplemented ration. Stratum corneum from both groups was isolated and spectral transmission recorded. Transmission, between 280–320 nm, was approximately 65% greater through stratum corneum obtained from control animals compared with that of BHT-treated animals. Further evidence of the biologic significance of this BHT effect upon stratum corneum absorption was obtained when stratum corneum was first removed by tape-stripping, the animals irradiated with 0.45 J/cm^2 of UVB, and epidermal ODC activity determined. BHT provided the usual inhibition of ODC activity induction in nonstripped animals, but ODC activity induction in BHT-treated, tape-stripped animals was restored to levels that did not significantly differ from controls. The protective effect exhibited by the stratum corneum could not be attributed to BHT-induced alteration of physical dimension, as neither the thickness of stratum corneum nor the number of stratum corneum layers, as determined from measurement of NaOH-distended frozen sections, differed from controls. Although the mechanism remains obscure, these data support the contention that systemically administered BHT results in diminished levels of UV radiation reaching potential epidermal target sites and delimits a large component of the photoprotective effect to the stratum corneum. *J Invest Dermatol 87:343–347, 1986*

Orally ingested antioxidants have been shown to provide systemic protection from UV radiation-induced erythema and photocarcinogenesis, the latter exhibited by decreased tumor multiplicity and increased time of tumor development following carcinomatous transformation [1–4]. Of the mixture of antioxidants previously used, butylated hydroxytoluene (BHT) has proved to be the most effective in providing this photoprotection [2,5]. Prefeeding BHT also significantly decreases UV radiation induction of epidermal ornithine decarboxylase (ODC) activity [5]. ODC catalyzes the rate-limiting step in the synthesis of the polyamines putrescine, spermine, and spermidine from the amino acid ornithine. Changes in polyamine levels reflect proliferative activity in cells, and ODC activity is elevated in malignant skin tumors. The degree of ODC stimulation has been related to the degree of malignant change [6,7]. Although it now appears induction of ODC activity lacks specificity as a marker for tumor production [8,9], it is at least an early event closely associated with the process. Whereas the induction of ODC activity may not be responsible for malignancy, the failure to control ODC production is consistent with carcinomatous transformation [10] and thus is a useful marker for tumor-promoting or -inhibiting agents.

While the mechanism(s) of BHT's photoprotective properties remain obscure, previous efforts to elucidate its action have pointed to a specific effect toward UV radiation insult. BHT did not inhibit induction of ODC activity by topically applied 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [5]. Forward scattering of UV radiation by the epidermis was significantly less in BHT-fed animals, which would indicate that increased absorption is a likely mode of action [11]. The BHT levels have been measured in skin, but maximal levels accruing would absorb no more than 1% of incident UV radiation [11]. The mean thickness of the skin does not change after feeding BHT [11], and it has been shown biochemically that BHT does not induce epidermal proliferation [12]. Because radiolabeled BHT accruing in the skin of experimentally fed animals has been found to represent nonmetabolized, nonconjugated BHT, it is unlikely that BHT present in skin reacts with any epidermal constituent to increase its absorptive characteristics [12].

More recently, it has been proposed that BHT present in the stratum corneum alters the chemical or physical properties of keratin, resulting in an increased absorption of UV radiation [12]. Herein we report the findings from our studies examining the possibility that the photoprotective effects of BHT reside in the stratum corneum.

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Reprint requests to: Homer S. Black, Ph.D., Photobiology Laboratory, Building 203, Veterans Administration Medical Center, 2002 Holcombe Boulevard, Houston, Texas 77211.

Abbreviations:

BHT: butylated hydroxytoluene
ODC: ornithine decarboxylase
SPF: sun protection factor
TPA: 12-*O*-tetradecanoylphorbol-13-acetate

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MATERIALS AND METHODS

Animals and Diets One hundred and ten albino, female hairless mice (SKH-HR-1), 3–4 months of age, were housed under a 12-h light–dark regimen at 21–23°C. Two groups of 55 animals each received a commercial mouse meal (Wayne Lab-blox, Continental Grain Co., Chicago, Illinois) supplemented by weight as follows: BHT—0.5%; control—no supplement. The mice were fed the respective diets ad libidum for 2 weeks prior to ODC, optical transmission, or stratum corneum thickness studies.

Optical Transmission Eight mice from each diet group were used to determine the effect of BHT feeding on the transmission of UV radiation through the stratum corneum. Mice were sacrificed by cervical dislocation and a flap of dorsal skin was excised and heat treated for 28 s at 55°C. The epidermis was then separated by blunt dissection from the dermis in approximately 2 × 3 cm sheets and floated (stratum corneum up) on 0.02% trypsin in 0.1 M sodium phosphate buffer at pH 7.2 for 2 h under N_2 atmosphere. These sheets were then rinsed once and refloated on 0.9% NaCl in 0.1 M sodium phosphate buffer, pH 7.2, overnight, again under N_2 atmosphere. An amorphous residue was gently removed from each stratum corneum, the sheets mounted bottom side down on a quartz slide, and visible water carefully blotted dry. Transmission scans, between 250–400 nm wavelengths, were recorded for 3 separate 12.6-mm^2 areas of each stratum corneum sheet using a Bausch and Lomb Spectronic 505 recording spectrophotometer. An average transmission spectrum for stratum corneum of animals from each diet group was constructed. The areas under each average spectrum were obtained by performing a numerical integration using a quadratic approximation for each 10-nm interval. The estimates were summed to obtain the areas under the curves between the wavelengths of interest. Spectral characteristics of control stratum corneum compare favorably with those obtained by others [13].

Ornithine Decarboxylase Assay Forty-two animals from each of the diet groups were used in a study to determine the relative contribution of stratum corneum absorbance to BHT's inhibitory effect upon induction of ODC activity. The stratum corneum of 21 animals from each diet group was stripped by applying multiple pieces of Scotch 3M Magic Transparent Tape to the dorsal surface of each animal (sparing the head) and slowly pulling them off together in one sheet. This technique we determined to be effective in removing most of the stratum corneum present (Fig 1). The remaining 21 animals from each diet group were not stripped and served for comparison with the stripped animals. All animals were then irradiated and their epidermides assayed for ODC activity.

The UV radiation source consisted of 2 Westinghouse fluorescent FS-20 sunlamps. These lamps emit between 280–420 nm, with principal emission from 290–320 nm, and peak emission at 313 nm. Within 10 min after stripping, mice were irradiated in a 7 × 12 inch open-top plastic cage without restraints. The UV source was positioned 13 cm from the dorsa of the animals. Total dose administered to the mice was equivalent to 0.45 J/cm^2 as measured with a calibrated circular thermopile and microvolt meter.

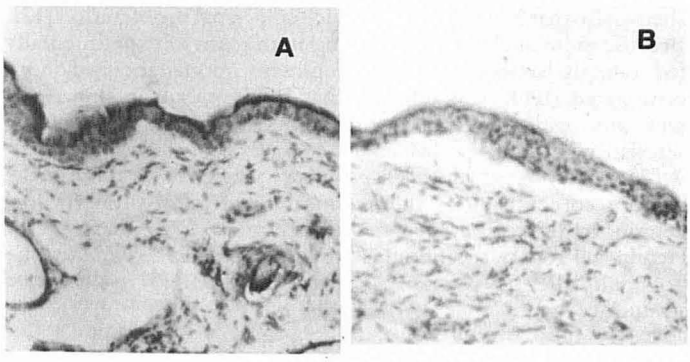

Figure 1. H & E stained sections of hairless mouse skin with (*A*) stratum corneum intact, and (*B*) stratum corneum stripped. Stratum corneum was stripped by application and removal of confluent strips of transparent tape. × 100.

Mice were sacrificed by cervical dislocation 28 h postirradiation. A flap of dorsal skin was excised and excess subdermal tissues scraped away. All manipulations of the skin were conducted on iced glass plates unless specified otherwise. The epidermis was scraped from the dermis after a 28-s 55°C heat treatment [14].

Epidermal preparations from 3 mice were pooled and homogenized in 3.2 ml of 50 mM sodium phosphate buffer (pH 7.2), containing 0.1 mM pyridoxal phosphate and 0.1 mM EDTA. The homogenates were centrifuged at 30,000 *g* for 30 min to give a soluble supernatant [15].

Supernatant ODC activity was determined by measuring the release of $^{14}CO_2$ from DL-[1-^{14}C]ornithine hydrochloride (sp act 59 mCi/mmol, Amersham Corporation, Arlington Heights, Illinois). Individual assay mixtures contained 35 mM sodium phosphate (pH 7.2), 0.2 mM pyridoxal phosphate, 4.0 mM dithiothreitol, 1.0 mM EDTA, 0.4 mM L-ornithine with 0.5 μCi of DL-[1-^{14}C]ornithine hydrochloride, and 100 μl of epidermal extract in a final volume of 0.25 ml. The mixture was incubated at 37°C for 60 min in rubber-stoppered tubes with center well, each containing 0.2 ml hyamine hydroxide [15]. The reaction was stopped by addition of 0.5 ml of 2 M citric acid and incubated for an additional 60 min. The center well was then transferred to a counting vial containing 10 ml of toluene-based scintillation fluid and 2.0 ml ethanol. Radioactivity was measured in a Beckman LS-230 liquid scintillation counter with 92% ^{14}C counting efficiency. All assays were conducted in quadruplicate and contained between 0.3–0.6 mg protein as determined by the method of Lowry et al [16]. Blank assays contained 100 μl H_2O. Animals were sacrificed and ODC activity routinely determined between 1 PM and 5 PM. Measurements of ODC activity on stratum corneum-stripped and nonstripped animals from each diet group were statistically compared using a regression model by which the mean was adjusted to compensate for incomplete block design. In all comparisons, significance was based on 2-tailed tests with p values of 0.05 or smaller.

Direct effects of stratum corneum stripping on ODC activity were also examined. Stratum corneum from 3 nonirradiated animals of each diet group were stripped as above and epidermal ODC activity measured 28 h later.

Histology and Stratum Corneum Measurements Three sheets of stratum corneum (used for the optical transmission studies) from each diet group were fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin, transverse sections of 5-μm thickness prepared, and stained with hematoxylin and eosin (Fig 2). The thickness of the stratum corneum was measured in 15 high-power fields (400×) at 140-μm intervals along the length of each stratum corneum.

In order to swell and count the layers of the stratum corneum, dorsal skin from 2 animals from each diet group was excised, embedded in Ames O.C.T. compound, frozen, and sectioned at 8-μm thickness. These sections were glass mounted, fixed for 30–45 min in 70% ethanol, and stained for 1 min with 0.1% methylene blue. The slides were then flooded with 0.1 N NaOH, drained, coverslipped, and examined microscopically to count layers in approximately 30 fields where sufficient swelling occurred to discern each layer of the stratum corneum (Fig 3) [17].

RESULTS

Average transmission spectra of stratum corneum from animals fed BHT and control diets are shown in Fig 4. Using the American Numerical Integration Procedure the areas under the curves for BHT- and control-fed animals over the UV spectrum, 250–

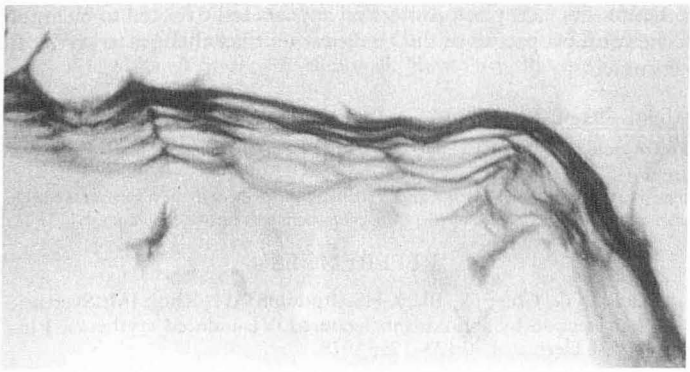

Figure 2. H & E stained section of isolated stratum corneum used for optical transmission and stratum corneum thickness determinations. Sheets of stratum corneum were isolated and floated on physiologic saline under nitrogen atmosphere prior to study. × 400.

400 nm, were 65 and 103 (units), respectively. For wavelengths 280–320 nm, the areas were 13.9 for BHT and 23 for control. These data indicate that approximately 65% more erythemic radiation would be required to elicit a similar response in BHT-fed animals as that which occurs in controls.

Further evidence of the biologic significance associated with the BHT effect upon stratum corneum absorbance is reflected in Table I, in which mean values of ODC activity from stratum corneum-stripped and nonstripped animals are compared. As previously reported [5], BHT feeding resulted in marked inhibition (approximately 70%) of UV radiation-induced ODC activity in nonstripped animals. However, when the stratum corneum of BHT-fed animals was stripped away prior to UV treatment, ODC activity was not significantly different from that of the UV radiation-induced nonstripped control ($p > 0.17$). Although UV radiation-induced activity for BHT-fed, stratum corneum-stripped animals was significantly different from that of stripped controls ($p < 0.02$), neither stripped treatment differed significantly from the nonstripped control ($p > 0.17$).

The effects of mechanical irritation on induction of ODC activity have been well documented [17]. However, at the 28-h postirradiation time interval of the current study, no ODC activity was measurable in tape-stripped, nonirradiated mice fed either diet (Table I). Thus, mechanical irritation could not account for any of the effects attributed to differences in stratum corneum transmission. Nor could transmission differences between stratum corneum derived from BHT-fed and control animals be attributed to changes in thickness or numbers of layers of stratum corneum (Table II), as there were no significant differences in these parameters ($p > 0.46$ and 0.21, respectively).

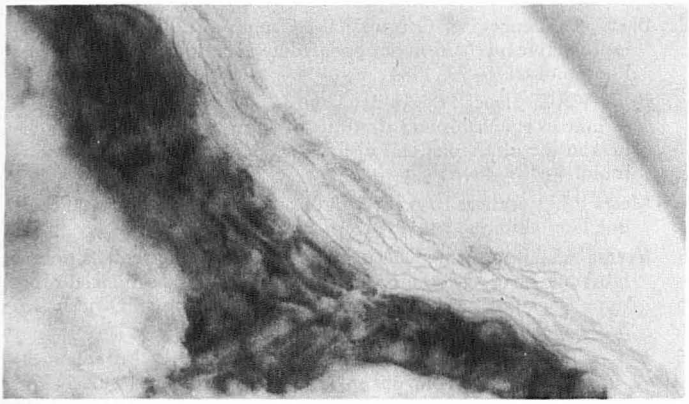

Figure 3. NaOH-distended stratum corneum of hairless mouse skin. Frozen sections were fixed in 70% ethanol, stained with methylene blue, and treated with 0.1 N NaOH to swell the layers of the stratum corneum such that individual layers could be counted. × 400.

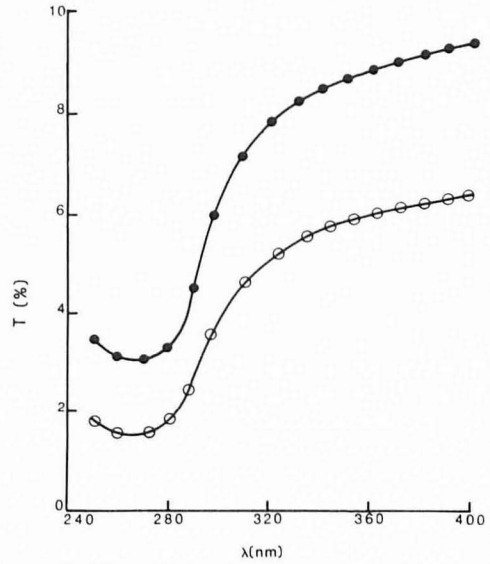

Figure 4. Average transmission spectra for stratum corneum from animals prefed BHT and control diets. Three scans on each of 8 samples of stratum corneum were averaged and areas under each average curve calculated for the respective treatment groups. *Closed circles*, control; *open circles*, BHT.

DISCUSSION

Previous studies have shown that epidermides from BHT-fed animals absorb UV radiation more strongly than nonsupplemented animals, and the sun protection factor (SPF) calculated from forward-scattering transmission scans was in good agreement with the erythema protection index of 2.0 reported earlier [1,11]. It became apparent that a diminution of UV radiation penetrating the epidermis could account for BHT's inhibitory effects upon all 3 observed biologic responses, i.e., photocarcinogenesis, erythema, and ODC activity. Direct measurement of epidermal levels of resident BHT, however, indicated that the agent, per se, would absorb no more than 1% of the incident UV radiation [11]. Nor did it appear that increased epidermal absorption could be explained by changes in the physical dimensions of the epidermal layer—as might be associated with BHT-induced hyperplasia reported for other types of epithelial cells [18].

The observation by Peterson et al [5] that systemically admin-

Table I. Influence of Butylated Hydroxytoluene (BHT)-Modified Stratum Corneum on UV Radiation-Induction of Ornithine Decarboxylase

Treatment	Mean[a]
Irradiated[b]	
Control (nonstripped)[c]	1.27[d] ± 0.19
Control (stripped)[e]	1.57 ± 0.18
BHT (nonstripped)	0.40 ± 0.07
BHT (stripped)	0.93 ± 0.34
Nonirradiated	
Control (stripped)	0.00
BHT (stripped)	0.00

[a] Adjusted mean ± SEM.
[b] Dose of 0.45 J/cm² administered 28 h prior to ODC assay.
[c] Intact stratum corneum.
[d] nmol $^{14}CO_2$/mg protein/h.
[e] Stratum corneum removed by tape-stripping. Animals were tape-stripped immediately prior to irradiation or at an analogous time for nonirradiated groups.

Table II. Stratum Corneum Measurements

Treatment	Thickness (μm)[a]	Number Cell Layers[b]
Control	20.1 ± 6.8	7.2 ± 1.1
Butylated hydroxytoluene (BHT)	20.7 ± 4.7	8.1 ± 1.5

[a]Mean ± SD; n = 45 measurements from biopsies of 3 animals in each group.
[b]Mean ± SD; n = 35 and 34 measurements from biopsies of 2 animals from control and BHT groups, respectively.

istered BHT inhibited the induction of ODC activity by UV radiation but not by TPA, the latter a potent tumor promoter, supports the contention that BHT's effects on skin represent a rather specific response to UV radiation insult. The facts that most polyamines are localized within the basal layer of epidermis and therefore the most likely site of ODC activity [19], and that the mouse epidermal layer is only about 2 cells thick, suggest that the protective effect resides in the stratum corneum. Gange and Mendelson [20] have recently shown that ODC activity can be used to determine the efficacy of topically applied sunscreens, and therefore it seemed reasonable that ODC activity might serve as a sensitive biologic actinometer to measure photoprotective effects localized in the suprabasal layers. Using their method, dietary BHT provides a protective effect equivalent to an SPF of 3.2. That BHT feeding results in increased stratum corneum absorption is apparent from the transmission scans depicted in Fig 4. The importance of dose diminution, its biologic consequences, and the delimitation of this effect to the stratum corneum is demonstrated by the ability to restore ODC activity in BHT-fed animals simply by removing the stratum corneum prior to UV radiation treatment (Table I).

Further evidence that BHT did not produce its inhibitory effect through influence upon the physical dimensions of the protective layer is provided by direct measurement of stratum corneum thickness in hematoxylin-eosin stained sections used in the transmission studies, and by comparing numbers of stratum corneum layers in NaOH-distended preparations.

It should be noted that ODC activity in stratum corneum-stripped, BHT-fed animals was less than that which occurred in stripped controls ($p < 0.02$), i.e., stratum corneum removal did not completely abate BHT's photoprotection. This finding suggests that either the single tape-stripping technique is not completely effective in removing all stratum corneum, or that BHT exerts a lesser protective effect in the malpighian layers, where it is undoubtedly present during keratin synthesis.

How might BHT alter the optical properties while not changing the physical dimensions of a tissue? If BHT elicits its protective response by virtue of its antioxidant properties, it is possible that during normal maturation processes sulfhydryl moeities are protected from oxidation, resulting in fewer disulfide cross-links. Certainly the degree of cross-linking has long been known to alter x-ray diffraction patterns of keratin [21] and it would be expected that optical properties are altered as well. Furthermore, the oxidation of 2 sulfhydryl groups to a disulfide bond in the stabilization of keratin generates a molecule of water. Increased sensitivity of hydrated skin to sunlight is well documented, and recently it has been shown that the degree of hydration of stratum corneum markedly affects its transmission characteristics [13,22,23]. Thus, it is conceivable that by interfering in normal oxidation of keratin, BHT could give rise to the observed optical changes of stratum corneum. Ancillary experiments show that BHT's protective effect upon induction of ODC activity in the hairless mouse is absent 1 week after discontinuing the dietary supplement— a time interval consistent with stratum corneum turnover.

Although the mechanism of BHT's photoprotective properties remains unknown at present, it has been demonstrated that a major component of this protective effect resides in the stratum corneum. Further, photoprotection appears to be related to changes in chemical properties of this tissue rather than changes in physical parameters.

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